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(54) Title: STEROID MODIFIED OLIGONUCLEOTIDES

(57) Abstract

Oligonucleotides modified at their backbones by the attachment of at least one steroid are described. The modified oligonucleotides anchor in the cell membrane to serve as a probe and to provide therapeutic activity.

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STEROID MODIFIED OLIGONUCLEOTIDES

GRANT REFERENCE

FIELD OF INVENTION, BACKGROUND AND PRIOR ART

         This invention relates to oligonucleotides
15 modified by a pendant steroid group. More
particularly, the present invention is related to
steroid modified oligonucleotides and a method of
using the modified oligonucleotides as antiviral
agents.

20   The pioneering work of Zamecnik and
Stephenson, Proc. Natl. Acad., 75:280-284 (1978), on
antiviral activity of oligonucleotides and Miller and
Ts'o, on the chemistry and biochemistry of non-ionic
analogues (Barrett, et al., Biochem., 13:4898-5
25 (1974) and Jayaraman, et al. Proc. Natl. Acad. Sci.
USA, 78:1537-1541 (1981)) has stimulated extensive

research directed at the therapeutic potential of
nucleotide polymers. Oligonucleotide analogues with
methylphosphonate, Miller, et al., _Biochemie_, <u>67</u>:769-
776 (1985), Agris, et al., Biochem., <u>25</u>:6268-6275

5   (1986), Smith et al., <u>Proc. Natl. Acad. Sci. USA</u>,
<u>83</u>:2787-2791 (1986), and Sarin, et al., <u>Proc. Natl.
Acad. Sci. USA</u>, <u>85</u>:7448-7451 (1988);
phosphorothioate, Matsukura, et al., <u>Proc. Natl.
Acad. Sci. USA</u>, <u>85</u>:7079-7083 (1988); and

10 phosphoramidate, Agrawal, et al., <u>Proc. Natl. Acad.
Sci. USA</u>, <u>85</u>:7079-7083 (1988), backbones as well as
natural type oligonucleotides, Zamecnik, et al.,
<u>Proc. Natl. Acad. Sci. USA</u>, <u>83</u>:4143-4146 (1986), and
a polylysine conjugate, Goodchild, et al., <u>Proc.

15 Natl. Acad. Sci. USA</u>, <u>85</u>:5507-5511 (1988), have now
been reported to inhibit viral replication in cell
culture. The viruses studied in this context include
Rous sarcoma virus, Samecnik and Stephenson, <u>Proc.
Natl. Acad.</u>, <u>75</u>:280-284 (1978); simian virus, Miller,

20 et al., <u>Biochemie</u>, <u>67</u>: 769-776 (1985); vesticular
stomatitis virus, Agris, et al., Biochem., <u>25</u>:6268-
6275 (9186) and Lemaitre, et al., <u>Proc. Natl. Acad.
Sci. USA</u>, <u>84</u>:648-652 (1987); human immunodeficiency
virus (HIV), Sarin, et al., <u>Proc. Natl. Acad. Sci.

25 USA</u>, <u>85</u>:7448-7451 (1988), Matsukura, et al., <u>Proc.
Natl. Acad. Sci. USA</u>, <u>84</u>:7706-7710 (1987), Agrawal,

et al., <u>Proc. Natl. Acad. Sci. USA</u>, <u>85</u>:7079-7083

(1988), Aamecnik, et al., <u>Proc. Natl. Acad. Sci. USA</u>,

<u>83</u>:4143-4146 (1986), and Goodchild, et al., <u>Proc.</u>

<u>Natl. Acad. Sci. USA</u>, <u>85</u>:5507-5511 (1988); herpes

5   simplex virus, Smith, et al., <u>Proc. Natl. Acad. Sci.</u>

<u>USA</u>, <u>83</u>:2787-2791 (1986); and influenza virus,

Zerial, et al., <u>Nuc. Acids. Res.</u>, <u>15</u>:9909-9919

(1987).

The concept underlying this work is that an

10 oligonucleotide complementary to a unique segment of

a viral genome, or an RNA derived from it, may

selectively disrupt processes dependent on that

segment by hybridization. This rationale is

supported by a variety of experiments with cell free

15 systems or with cells to which "antisense"

polynucleotides have been inserted by microinjection

or transfection, C.A. & Cohen, J.S., <u>Cancer Res.</u>,

<u>48</u>:2659-2668 (1988). However, the actual mechanisms

by which oligonucleotides and their analogs function

20 as inhibitors in cell cultures are still far from

clear. In particular, little is known about the

interaction of the oligomers with cell membranes or

the locus of their reactions within cells. It

appears that non-ionic oligomers, such as the methyl

25 phosphonate analogues diffuse passively through cell

membranes.

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S.E. Clare has synthesized oligonucleotides possessing one or more 2,2,2,-trichloral-1,1-dimethylethyl (TDCME, lipophilic) group of the phosphorous atom in the chain and show that this

5   group on one strand with proper stereochemistry can inhibit cleavage of the opposite strand by a restriction endonuclease and that the same group on a template will inhibit synthesis of the complementary strand by the Klenow enzyme. S.E. Clare also

10   demonstrated a single modification 5' to dGNAd (CG) octamer by TDCME group prevents the B to Z conformational transition. S.E. Clare, Ph.D. Dissertation, Northwestern University, Evanston, Illinois (1987).

15   The present invention is related to a family of oligonucleotides modified at the backbone so that the oligonucleotide may anchor at the cell membrane to provide antiviral effects. The present invention describes a family of oligonucleotides with

20   a modification designed to anchor the oligomer, at least transiently at the cell membrane, to inhibit HIV-1 in cell culture. Fatty substances have been selected as an anchor for the oligonucleotide, and without being limitative, steroids such as

25   cholesteryl have been selected as the preferred

anchor since they are highly hydrophobic and cell
membranes have an abundance of this steriod. The
compounds may also have anti-sense activity.

The cholesteryl is a large lipophilic

5   group, much larger than the TDCME group. In
principle, such pendent groups, when linked
covalently to the internucleotide phosphorous atoms,
have potential as lipophilic centers to enhance the
interaction with membranes, to alter partitioning of

10 oligonucleotides within cells, to inhibit certain
enzymatic reactions and to influence the stability of
hybrids joined with natural polynucleotides.
Cholesteryl is a component of any biological membrane
and interacts with other lipids. The AIDS virus,

15 HIV, is distinguished by an unusually high
cholesteryl content in the lipid membrane. Early
model studies by Finean, Experientia, 9:17-19 (1985),
suggested that the cholesteryl molecule is capable of
formation of a stabilizing complex with the

20 phospholipid molecule. The hydrocarbon chain of the
cholesteryl is bound to the parallel portion of the
phospholipid chain by Van DeWall forces. Recent
studies employing a variety of techniques indicated
that the major forces may involve the hydrophobic

-6-

portion of the lipid molecules. Therefore,
cholesteryl is a preferred modifying group for
oligonucleotide interaction with cells.

5   SUMMARY OF THE INVENTION

         The invention is concerned with
oligonucleotides that are modified to anchor the
oligomer at the cell membrane or eventually,
10 intranuclearly, so that the oligonucleotide may serve
as a probe and provide therapeutic activity.
         More specifically, the invention provides a
pharmaceutical composition including an
oligonucleotide conjugated to a steroid for
15 increasing the antiviral activity of the compound.
One aspect of the present invention provides
oligonucleotide compounds represented by the
following structural formula:

Formula I

RO——————O—————B
（糖環構造）

$$O = P - N - A - N - C - O - Cholesteryl$$

```
             O                    R    O
             |                    |    ||
         O = P  -  N  -  A  -  N  - C  -  O  -  Cholesteryl
             |     |              |
             O     R              R
             |
            NUC
```
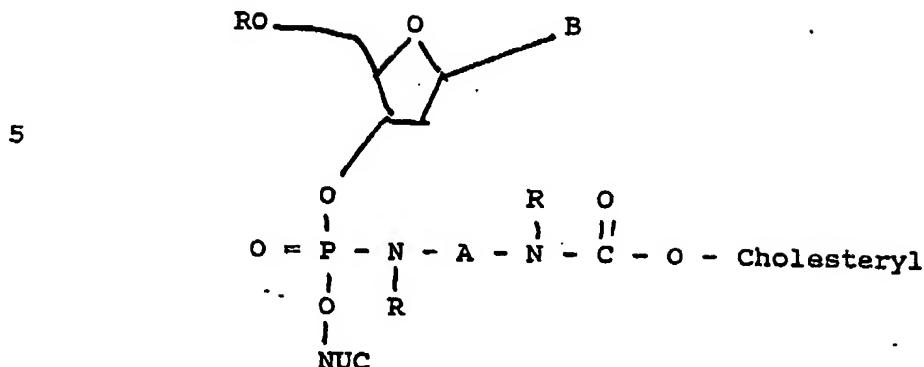
wherein A = aliphatic alkyl or branched
aliphatic alkyl or a heteroatom containing an alkyl
(branched) chain of 2 to 18 carbon atoms, preferably
CH_2, R = H and lower alkyl up to 12 carbon atoms,
preferably methyl; NUC refers to an oligonucleotide
which may be a deoxyribonucleotide or a
ribonucleosite. Preferred nucleotides are
ethymidine, deoxyadenosine, deoxyguanosine and
deoxycytidine. The nucleotides are connected
respectively to the phosphorous through their 3' and
5' oxygens, B is a purine or pyrimidine base (such as
Thy, Cyt, Gua, Ade).

Cholesteryl has been selected as the
preferred anchol because it is highly hydrophilic and
found in cell membranes. The cholesteryl modified
oligonucleotides of the present invention have been

found to inhibit HIV-1 in cell culture. The location

of the insertion of the cholesteryl anchor on the

oligonucleotide may be varied and is not dependent on

sequence. However, other steriods are efficacious

5

DETAILED DESCRIPTION OF THE INVENTION

The novel compounds of Formula I can be

prepared by convenient procedures for introducing a

10　cholesteryl group at any desired internucleoside

phosphorous in the course of synthesizing an

oligonucleotide. The cholesteryl may be linked to an

oligonucleotide as a substituent at either the 3'-O

or 5'-O terminus.

15　　　　　Processes for preparing the novel compounds

of Formula I are generally described by equations A

and B.

A.

20

(1)　$NHR(CH_2)_nNHR$ + $\overset{O}{\overset{\|}{X C O}}$-cholesteryl $\longrightarrow$ $NHR(CH_2)_n\overset{R}{\overset{|}{N}}\text{-}\overset{O}{\overset{\|}{C O}}$-cholesteryl

(2)　$NHR(CH_2)_n\overset{R}{\overset{|}{N}}\text{-}\overset{O}{\overset{\|}{C O}}$-cholesteryl + $R'O\overset{O}{\overset{\|}{P}}\text{-}OR''$ + CCl_4 $\longrightarrow$

25　　　　　　　　　　　　　　$R'O\overset{O}{\overset{\|}{P}}OR''$
　　　　　　　　　　　　　　　　　$\overset{|}{\underset{R}{N}}(CH_2)_n\overset{R}{\overset{|}{N}}\text{-}\overset{O}{\overset{\|}{C O}}$-cholesteryl

<u>B</u>.

$$(3) \quad R'O\overset{\overset{O}{\|}}{\underset{\underset{H}{|}}{P}}OR'' + NHR(CH_2)_nNHR + CCl_4$$

$$\longrightarrow \quad R'O\overset{\overset{O}{\|}}{\underset{\underset{R}{|}}{P}}OR''$$
$$\underset{N(CH_2)_nNHR}{}$$

$$(4) \quad R'O\overset{\overset{O}{\|}}{\underset{\underset{\underset{R}{|}}{N(CH_2)_nNHR}}{P}}OR'' \quad + \quad X\overset{\overset{O}{\|}}{C}O\text{-cholesteryl}$$

$$\longrightarrow \quad R'O\overset{\overset{O}{\|}}{\underset{\underset{\underset{R}{|}}{N(CH_2)_n}}{P}}OR'' \quad \overset{\overset{O}{\|}}{\underset{\underset{R}{|}}{N}}CO\text{-cholesteryl}$$
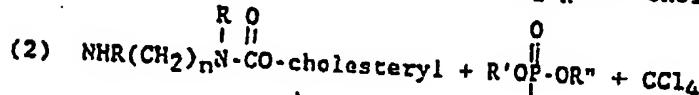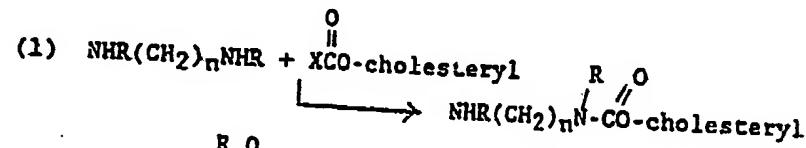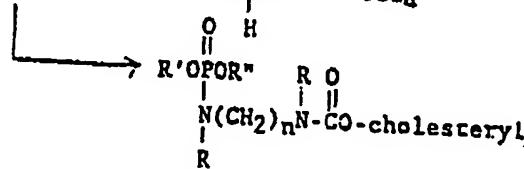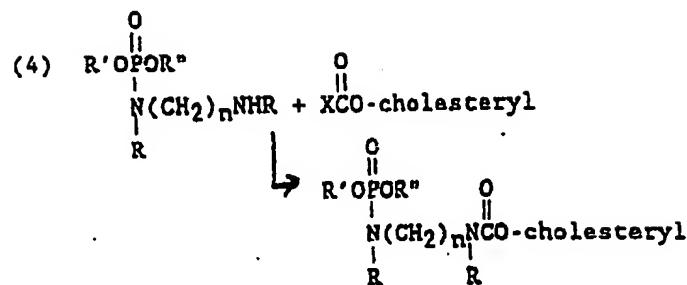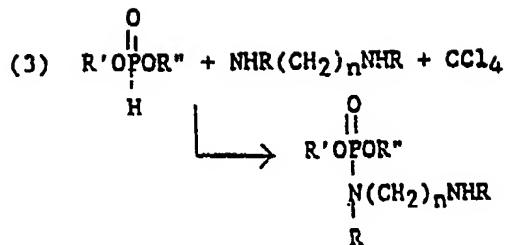
In the reactions, X = Cl- and p-nitrophenoxy, n = 2 and 6, and R = H and methyl. The

5　reactions for linking the amines to phosphorous are based on the general procedure of Froehler, B.C., <u>Tet. Lett.</u>, <u>27</u>:5575-5578 (1986) for generating P-N bonds in oligonucleotide derivatives. The article is incorporated by reference.

10　　　　Equation A avoids side reactions involving condensation at both nitrogen atoms of the diamine to form bis-phosphoramidates. Procedures for preparing fifteen compounds of Table I with the cholesteryl anchor are described as follows: phosphodiester links

15　were formed by cyanoethyl phosphoramidite chemistry described in the standard synthesis protocol provided

by the manufacturer of the synthesizer, for example,

Biosearch 8600, Biosearch, Inc., San Raphael,

California. Chain extension by hydrogen phosphonate

chemistry is described by Froehler, et al., Tet.

5   Lett., 27:469-472 (1986) and Froehler, et al., Nuc.

Acids. Res., 14:5399-5407 (1986). Phosphorothioate

functional groups are added by the procedure of

Froehler, et al., Tet. Lett., 27:5575-5578 (1986).

10   Experimental Procedure

2-(Cholesteryloxycarbonylamino)ethylamine.

        Cholesteryl chloroformate (2g) in

dichloromethane (6 ml) was added dropwise to a

15   solution of ethylenediamine (2.5 ml) in

dichloromethane (6 ml) and pyridine (6 ml). The

mixture was stirred for two hours; then the solvent

was removed under vacuum and the residue was

partitioned between water (150 ml) and

20   dichloromethane (150 ml). The organic layer was

washed with water, dried (Na_2SO_4), and concentrated

to give the title compound; 1.6 g (76%), mp 149-155

degrees Centigrade. Recrystallization from

cyclohexane afforded crystales melting at 152-155

25   degrees Centigrade; Rf on silica (CHC_{13}/MeOH, 1/1

v/v) 0.15; positive ninhydrin test. Anal. Calcd for $C_{30}H_{52}N_2O_2$; C, 76.22; H, 11.09; N, 5.93. Found: c, 75.96; H, 11.10; N, 5.86.

5   Preparation of Cholesteryl-Modified Dinucleoside
    Monophosphate on CPG Support.

            Internucleoside cholesteryl side chains
    were linked to phosphorous via phosphoramidate bonds
    (adaption of procedure of Froehler). The preparation
10 of d-DMT-ibG$_c$ibG-CPG. is representative. A sample of
    DMT-ibG linked through the 3'-O to a controlled-por-
    glass support (Biosearch) (250 mg. 8 micro moles of
    DMG-ibG) was placed in a Glencoe Gastight syringe (10
    ml) equipped with a plug of glass wool at the inlet.
15 Reactions and washings were carried out by drawing in
    and ejecting the desired solutions. Thus, the DMT
    groups were removed with dichloroacetic acid (2.5% in
    CH_2Cl_2; the support was washed repeatedly with
    C_5H_5/CH_3CN (1/4), coupling was effected by drawing in
20 together DMT-ibG-hydrogen phosphonate (80 mg., 0.1
    mmol, in 1.2 ml CH_3CN/C_5H_5) and trimethylacetyl
    chloride (65 micro L, 0.5 micro moles, in 1.2 ml
    CH_3CN/C_5H_5, 1/1 v/v; 2 minutes), and the support was
    washed well with CH_3CN/C_5H_5. A solution of
25 cholesteryloxycarbonylaminoethylamine (250 mg, 0.5
    mmol) in CCl_4 (5 ml) and C_5H_5 (2 ml) was then drawn

into the syringe and after 0.5 hours, the solution
was ejected and the solid was washed well with CH_3CN.
Appropriate portions were then transferred to a
cartridge for extension by machine synthesis

5   (Biosearch 8600 Synthesizer) or to a syringe for
manual synthesis.

Chain Extension. The oligonucleotide
chains were extended by conventional phosphoramidite
chemistry in constructing phosphodiester links and by

10   hydrogen phosphonate chemistry in building the
phosphorothioate derivatives. The manual procedure
used in adding a thymidine unit to DMT-ibG*G-CPG in
synthesizing compound 2 in Table 1 is representative
of one synthetic cycle utilizing a phosphoramidite

15   reagent.

The DMT(G*G) loaded CPG (30 mg, 1 micro
mole) was poured into a 1.0 ml Glenco Gas tight
syringe with a glass wool plug at the inlet. Washes
were effected by drawing up the desired amount of the

20   reagent, resuspending the support by brief hand
agitation, and ejecting the solution. The DMT
protecting group was removed by washing with
DCA/CH_2Cl_2 (2.5/100 v/v, 5.0 ml), and organ effluents
were pooled from subsequent spectroscopy (447 nm) and

25   calculation of the coupling efficiency. The support
was washed successively with C_5H_5N/CH_3CN (1/4, v/v, 1

X 0.5 ml), and CH_3CN (2 X 0.5 ml). Any unreacted
5'-OH groups were capped by drawing DMAP in C_5H_5N/THF
(0.3 M, 1/15, v.v, 0.5 ml) into the syringe followed
immediately by Ac_2O/THF (0.6 M, 0.5 ml). The mixture

5   was agitated for one minute, capping agents were
ejected from the syringe, and the support was washed
with C_5H_5N/CH_3CN (1/4, v/v, 1 X 5.0 ml) and CH_3CN (1X
0.5 ml). The phosphite internucleoside linkage was
oxidized to the phosphotriester linkage with I_2 in

10   C_5H_5N/THF H_2O (0.1 M I_2, 18/80/2, v/v/v, 0.5 ml) for
two minutes. The oxidant was ejected, and the
support was washed with C_5H_5N/CH_3CN (1/4, v/v, 3 X
1.5 ml) and CH_3CN (3 X 1.5 ml) to complete one
synthetic cycle.

15   For chain extension by hydrogen phosphonate
chemistry the DMT-ibG*ibG-cpg (1 micro mole loaded
dimer) was detritylate as in the previous case. A
solution of the DMT-nucleoside hydrogen phosphonate
(10 mg, about 15 micro moles, in CH_3CN/C_5H_5N (1/1,

20   v/v, 0.3 ml) was drawn into the syringe, which was
agitated for two minutes. The coupling agents were
ejected from the syringe, and the support washed with
C_5H_5N/CH_3CN (1/1, v/v, 0.5 ml), and CH_3CN (3 X 0.5
ml) to complete one synthetic cycle. Additional

25   couplings were performed by returning to the initial
wash and repeating the cycle. Oxidation following

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the final coupling step was performed by treatment
with 0.1 M sulfur in CCl_4/Et_3N (9/1, v/v) at room
temperature (two hours reaction). Procedures for
machine syntheses were similar.

5

Isolation of Oligonucleotides

The oligomers were removed from the syringe
or the synthesizer and warmed in a capped vessel with
concentrated NH_4OH at 55 degrees Centigrade for five

10 hours. The aqueous solution was then removed and
concentrated under reduced pressure to give the crude
oligonucleotide. This substance was chromatographed
on a C-18 column and the band corresponding to the
desired target oligomer was collected and

15 lyophilized.

Table 1. Properties of Oligonucleotides

compound		HPLC[a] min	TLC[b] R_f	PAGE[c] R_m	T_m[d] °C
1	ACACCCAATTCTGAAAATGG	12.2	0.26	0.64	60
	Cholestaryl Substituents				
2	ACACCCAATTCTGAAAATG*G	46.0	0.41	0.55	60
3	ACTCCGAAAGATAAAG*G	46.6	0.43	0.54	--
4	A*CACCCAATTCTGAAAATG*G	61.0	0.58	●	52
5	CAATTCTCAAAATG*G	46.5	0.54	0.64	46.5
6	$A_SC_SA_SC_SC_SC_SA_SA_ST_ST_SC_ST_SG_SA_SA_SA_SA_ST_SG_SG$	16.5	0.59	0.67	44
7	$A_SC_SA_SC_SC_SC_SA_SA_ST_ST_SC_ST_SG_SA_SA_SA_SA_ST_SG^*G$	40.2	0.61	0.64	47.5
8	$C_SA_SA_ST_ST_SC_ST_SG_SA_3A_3A_3T_3G_SG$	17.3	0.47	0.67	29
9	$C_SA_SA_ST_3T_SC_3T_SG_SA_3A_3A_3A_3T_SG^*G$	47.4	0.59	0.63	25
10	$C_ST_SG_SA_SA_SA_SA_3T_SG^*G$	15.6	0.52	0.76	--
11	$C_ST_3G_SA_SA_SA_SA_ST_6G^*G$	49.6	0.56	0.62	--
12	$G_SA_SC_ST_ST_ST_ST_SA_SG^*G$	45	0.60	0.79	--
13	$C_3T_SG_SA_3T_3T_3T_3T_SG^*G$	45.2	0.59	0.76	--
14	$A_SA_SA_SA_SG^*G$	47.5	0.66	0.62	--
15	$T_ST_ST_ST_ST_ST_ST_ST_3T_ST_ST_ST_ST_ST^*T$	50.0	0.60	0.65	16

In formulas * represents $O=P-NH(CH_2)_2NHCO_2$ Cholesteryl; s represents $O=P-S^-$, and + represents $O=PNH(CH_2)_3N(CH_3)_2$. Altered nucleotides in 7 are underlined.

5   [a]Elution time, Hewlett-Packard RP-C18 column (10 cm); 0.1 M triethylammonium acetate (pH 7.0), 1%/min acetonitrile gradient starting at 0% acetonitrile; 0.5 ml/min flow rate.

        [b]Thin layer chromatography on Merck silica 10 plates with propanol/ammonium hydroxide/water, 55/10/35 v/v/v.

        [c]Polyacrylamide gel electrophoresis in 20% crosslinked gel at pH 8.0, R_m is migration relative to bromophenol blue.

15   [d]T_m is the temperature at the midpoint for the maximum slope in a plot of A_{260} versus temperature in 0.1 M aqueous NaCl, 0.01 M Tris buffer at pH 7.0; total nucleotide concentration (base units) in approximately 10^{-4} M. In each case, the 20 complement is a phosphodiester strand equal in length to the modified oligomer.

        [e]The sample appeared as a broad streak starting at R_m 0.2. The complement for determination of T_m was poly d(A).

25

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Coupling efficiency introducing a
cholesteryl fragment to the compounds of Table 1
exceeded 50%.

As noted, phosphodiester links were formed
5   in compounds 2-5 by conventional cyanoethyl
phosphoramidaite chemistry as described in the
standard synthesis protocol provided by the
manufacturer of the synthesizer, Biosearch, Inc., San
Raphael, California. For compounds 6 - 15, the
10   chains were extended by hydrogen phosphonate
chemistry as described by Froehelr, et al., Tet.
Lett., 27:469-472 (1986) and Froehler, et al., Nuc.
Acids. Res., 14:5399-5407 (1986) the final oxidation
with sulfur to generate the phosphorothioate groups.
15   The compounds were characterized by HPLC,
TLC, PAGE, thermal disassociation curves for hybrids
formed with complimentary strands and by UV and NMR
spectroscopy. The NMR spectra exhibited the
characteristic peaks for phosphodiester,
20   phosphoramidate and phosphorothioate functional
groups. A proton NMR spectrum of compound 2 shows
the presence of the cholesteryl fragment. Further,
the hydrophobic nature of the cholesteryl-
oligonucleotides was shown by the HPLC data in Table
25   1. For example, the elution times for samples
analyzed by a reverse phase C-18 column increased

from 14 to 46 to 61 minutes for the series 1
(control), 2(one) cholesteryl, and 4(two)
cholesteryl, respectively. Susceptibility to
nuclease degradation was examined with compound 2.

5   In the presence of snake venom, phosphodiesterase, an
alkaline phosphatase, compound 2 was completely
hydrolyzed to the expected nucleosides and the
fragment corresponding to the terminal G*G.

        The data in Table 1 shows that the

10 introduction of a single cholesteryl fragment at a
terminal internucleoside position has only a minor
effect on the stability of the hybrid duplex as
measured by T_m values when compound 1 is compared
with 2; compound 6 compared with 7, and compound 8

15 compared with 9. Conversely, two cholesteryl
substitutents led to appreciable destabilization for
the 20-mer compound, compare compound 4 with 1. The
disassociation of complexes formed from equi-molar
quantities in modified and unmodified complementary

20 oligodeoxyribo-nucleotides were measured by changes
in adsorbents in 260 nm as a function of temperature.

        Additional compounds were made in
accordance with Equation B, wherein R = CH_3, N = 6, X
= $-OC_6H_5MO_2$. The following compounds were prepared:

25 16, TTTTTTTT#T; 17, T#TTTTTTTT; 18, TTTT#TTTTT;
and 19, CGCG#AATTCGCG, where # is O = P-N

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$(CH_3)(CH_2)CO_2$ cholesteryl. The procedures of
Froehler, B.C., <u>Tet. Lett.</u>, <u>27</u>:5575-5578 (1986) and
Marcus-Sekura, et al., <u>Nuc. Acids. Res.</u>, <u>15</u>:5749-5763
(1987) were followed in preparing phosphodiester

5   links in compounds with internal modifications to
avoid or minimize complications which could arise
from the premature formation of phosphodiester groups
in the course of the synthesis. As in the case of
the derivatives of ethylenediamine, introduction of

10 substituents at the terminal internucleoside links
had little effect on T_m values for the hybrids formed
in complementary sequence (T_m for 1:1 complexes with
poly d(A) in 0.1 M NaCl, pH 7: 28 degrees Centigrade
for compound 16, 27 degrees Centigrade for compound

15 17 and 28 degrees Centigrade for parent T_9T).
Conversely, modification of the centrally positioned
internal link led to significant destabilization (21
degrees centigrade T_m for compound 18). The mobility
of stereoisomers of compound 19 on HPCL differed

20 sufficiently to permit separation of the isomers.
The T_m for the duplexes formed from these self-
complementary modified strands (stereoisomers of
compound 15) were substantially lower than that for
the parent duplex (T_m:40 degrees and 45 degrees

25 Centigrade for the isomers as compared to 56 degrees
Centigrade for CGCGAATTCGCG; 0.1 M NaCl, pH 7.0).

The linking of cholesteryl to an oligonucleotide as a substituent at the 5'-O terminus is quite simple and can be shown by the following example.

5

Synthesis and characterization of cholesteryl-sTsGsG.

DMT(G) loaded CPG (88.2 mg, about 3 micro moles) was poured into 5 ml Glencoe gas tight syringe with a glass wool plug at the inlet. Washes were

10 effected by drawing up the desired amount of the reagent, resuspending the support by brief hand agitation and ejecting the solution. The support was initially washed with CH_3CN (4.5 ml X 3) and CH_2Cl_2 (4.5 ml X 2). The DMT protecting group was removed

15 by washing with DCA/CH_2Cl_2 (2.5/100, v/v, 10 ml). All of the orange effluents were pooled for subsequent spectroscopy (448 nm) and calculation of the coupling efficiency.

The support was washed successively with

20 pyridine/CH_3CH (1/4, v/v, 4.5 ml X 3), CH_3CN (4.5 ml X 4) and dry CH_3CN (4.5 ml X 6). The H-phosphonate solution (for G and T - 36 mg, about 0.06 micro moles in dry CH_3CH/pyridine, 1/1, v/v, 2ml); for cholesteryl H-phosphonate-42 mg., about 0.09 micro

25 moles in dry CH_3CN/pyridine, 1/3, v/v, 2.0 ml) and trimethylacetyl chloride solution (0.03 ml, about

0.351 micro moles in trimethylactyl chloride solution
(0.03 ml, about 0.351 micro moles in dry
CH_3CN/pyridine, v/v, 2 ml) were drawn into the
syringe, which was agitated for five minutes (but for
5   15 minutes for cholesteryl H-phosphonate coupling).
After each coupling, the reagents were ejected from
the syringe and the procedure was continued by
returning to the initial wash steps.

Oxidation, following final coupling step
10   and wash with dry CH_3CN (4.5 ml X 3), dry pyridine
(4.5 ml X 4) was performed with 0.1 58 in
CS_2/pyridine (1/1, v/v, 4.5 ml X 2), CH_3N (4.5 ml X
3), dry CH_3CN (4 ml X 3) and ether (5 ml X 4). After
drying the CPG-bound product was treated with 3.0 ml
15   concentrated NH_4OH at 55 degrees Centigrade for five
hours. Upon removal of NH_4OH by evaporation under
reduced pressure, CPG support was removed by
filtration; the filtrate freeze-dried overnight and
the product redissolved in 2.0 ml H_2O . For UV
20   spectroscopy, 10 micro liters of this solution were
added to 990 micro liters of H_2O.

HPLC data indicated about 50% of the
reaction mixture was the desired product.
Spectroscopic methods also confirm the desired
25   structure.

Compound of Formula I of the present

invention can also be utilized in a method for

hybridizing with a complementary sequence in a

solution under conditions conducive to the

5   hybridization. Typically, these conditions are

controlled by the complementary sequence. The

specific conditions needed for hybridization would be

known to one skilled in the art familiar with the

complementary sequence and environment for

10 hybridization.

The present invention further comprises the

method of modifying the backbone of an

oligonucleotide by the attachment of a fatty

substance, preferably cholesteryl so that it will

15 anchor into the cell membrane so that the modified

oligonucleotide will hybridize with the complementary

sequence. By anchoring into the cell membrane, the

oligonucleotide may provide diagnostic or therapeutic

activity. For example, the oligonucleotide compound

20 1 of Table 1 is complementary to the splice acceptor

for site at 5349-5368 in HIV-1 and has been shown to

inhibit replication of this virus in MOLT-3 cells by

Zerial, et al., Nuc. Acids Res., 15:9909-9919 (1987)

and Stein, et al., Cancer Res, 48:2659-2668 (1988).

25 The compounds shown in table 1 are structural

variations of the basic sequence of compound 1.

Compounds 2 - 15 were designed to provide information
on the antiviral properties of the cholesteryl
modified oligonucleotides and, specifically, on the
dependence of the antiviral activity on 1 (the number

5 of cholesteryl fragments incorporated in the backbone
chain); 2 (the nature of the main backbone section
e.g. phosphodiester venus phosphorothioate links), 3
(the length of the oligonucleotide) and 4 (the
sequence integrity of the oligonucleotide.

10   Samples of the oligomers were assayed in
the following test:

Assays for HIV-1 Inhibition.

          The inhibition of HIV-1 expression of H9 or
15 MOLT-3 cells in the presence of antisense
oligonucleotides was carried out by infecting 5×10^5
cells per ml with $2.5-5 \times 10^8$ virus particles of
HIV-1 strains HTLV-IIIB or HTLV-IIIC. Infection with
500-1000 virus particles per cell represents a

20 multiplicity of infection (MOI) of 0.5-1. HIV-1
infection of cells was carried out by simultaneous
addition of virus and cholesteryl modified oligomers
to the cells in culture. The cultures were incubated
in culture medium containing RPMI 1640, 10% (v/v)

25 fetal bovine serum, 2mM gultamine, and 250 micrograms
of gentamicin per ml, in a humidified atmosphere

containing 5% CO_2 at 37 degrees Centigrade. After
four days, the cells and supernatant were examined
for the level of HIV-1 expression by measuring
syncytia (MOLT-3 cells) and viral antigen expression

5   as well as cell viability. The number of syncytia
formed MOLT-3 cells were counted after triturating
the cells to obtain an even distribution of the
syncytia in the culture. The average number of
syncytia as obtained by counting several fields in

10   duplicate cultures. Cell viability was measured in
the presence of trypan blue, and the cells that
excluded the dye were counted as viable cells. HIV-1
antigen expression was measured in cells fixed in
methanol/acetone as described. Sarin, et al.,

15   Biochem. Pharmacol., 34:075-4078 (1985) and Sarin, et
al., J. Natl. Cancer Inst., 78:663-666 (1987). In
brief, the cells were pelleted and then resuspended
in phosphate-buffered saline (PBS) at a concentration
of 10^6 cells per ml. The cells were spotted on

20   toxoplasmosis slides, air-dried, and fixed in
methanol/acetone (1:1, v/v) for 15 minutes at room
temperature. The slides were next incubated with 10%
normal goat serum at room temperature for 30 minutes
and washed with PBS four times. HIV-1 p24 or P17

25   monoclonal antibody was added to each well and the
slides were incubated for 30 minutes in a humid

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chamber 15 37 degrees Centigrade. The slides were
then washed four times with PBS, incubated with
fluorescein isothiocyanate-labeled goat anti-mouse
IgG (Cappel Laboratories, Cochranville, Pennsylvania)
5 for 30 minutes at 37 degrees Centigrade, and then
washed with PBS overnight. The slides were
counterstained with Evan's blue, washed with PBS,
mounted with 50% glycerol, and examined with a Zeiss
fluorescence microsocpe. The percentages of cells
10 fluorescing in the oligomer-treated and untreated
cultures were compared. Inhibition of HIV-1
expression in the presence of oligomers was found to
be similar in both the H9 and the MOLT-3 cells.

Inhibition of HIV-1 expression and H9 and
15 MOLT-3 cells in the presence of cholesteryl modified
oligonucleotides was carried out and results shown in
Tables 2 and 3.

The data for the inhibition of formation of
syncytia, an expression of HIV proteins P17, P24 and
20 reverse transcriptase shown for compounds 1 - 5 in
Table 2 and compounds 6 - 16 in Table 3. The tables
show results in ID_{50} values for inhibition of
syncytia (concentration of an oligomer in micro
grams/ml. It gives 50% inhibition under the assay
25 condition) as an index.

The data from the tables describe favorable
conclusions. The activity of the parent
oligonucleotide, compound 1, is relatively low (ID50
less than 100). It appears that anchoring a
5   cholesteryl fragment to the oligonucleotide
significantly enhances the anti-HIV activity (from
ID50 greater than 100 to 10). Thus, the cholesteryl
provides steroid means conjugated to the
oligonucleotide for increasing the antiviral activity
10   of the oligonucleotide. Further, anchoring a second
cholesteryl fragment does not appear to be an
improvement because the second cholesteryl leads to a
reduction in activity relative to the
monocholesteryl-oligonucleotide. It appears that a
15   cholesteryl fragment to a phosphorothioate
oligonucleotide analog enhances the antiviral
property of the phosphorothioate derivative as shown
in comparisons between compounds 6 and 7, 8 and 8,
and 10 and 11. In the most favorable case, compound
20   7, the ID50 was reduced to 0.8 micrograms per
milliliter. With relatively large oligomers, those
having 15 to 20-mers, the activity of the
cholesteryl-oligonucleotides (natural phosphodiester
links) appears to be independent of the chain link
25   (compare compounds 2 and 5). A lack of dependence of
activity on link has also been shown for unmodified

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oligonucleotides in the 15-20 mer range.
Additionally, the activity of the cholesteryl
modified phosphorothioate derivates shows a downward
trend as the length of the oligomer is decreased.

5   Thus, The ID50 values increase from 0.8 for the 20-
mer (compound 7) to about 3.5 for the 10-15 mers
(compound 11 and 9), to 13 for the 6-mer (compound
14).

From Tables 2 and 3, it can also be

10 concluded that the anti-HIV activity of the
cholesteryl-modified oligonucleotides is not strongly
dependent on the nucleotide sequence. This
conclusion applies both to the phosphodiester and the
phosphorothioate cholesteryl derivatives (compared to

15 the data for compounds 2 and 3 has six mismatched
base sites; and the data for compound 11 with that
for compounds 12 and 13 which have 8 and 3
mismatched). For phosphorothioate derivatives, the
activity of all three 10-mers is essentially the same

20 although the sequence is different. Further, the
cholesteryl modified oligomers are not toxic to cells
even at concentrations far in excess of those that
lead to complete inhibition of the replication of
HIV. For all derivates the LB50 was greater than 100

25 micrograms per ml.

Additional Oligonucleotides having Steroids
Conjugated Thereto Possessing Antiviral Activity

　　　　Additional compounds and their antiviral

activity are set forth in Table 4. Activity is

5 reported as the average ID50 values (the

concentration in micrograms per ug/ml of oligomer

that leads to 50% inhibition of virus) determined by

syncytia formation and expression of the viral

proteins P17 and P24. The procedure is referenced in

10 Letsinger et al (1989) Proc. Natl. Acad. Sci. USA,

86:6553-6556. The abbreviations used in the Table

are set forth below.

15 ...NsN'... internucleoside phosphorothioate link, as in ..CsG..

20   --- C$_S$G ---

25
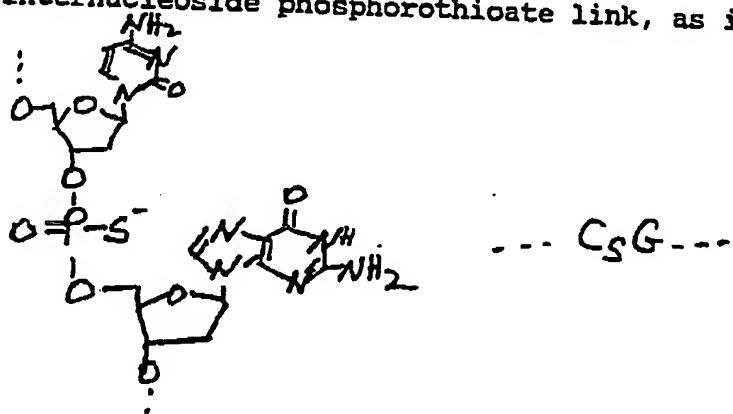
...N N'... internucleoside link with a cholesteryl group tethered
    chol   by a linker containing two carbon atoms, as in ..G G..
   chol

$O=\overset{\overset{O}{|}}{\underset{\underset{O}{|}}{P}}-NCH_2CH_2N-\overset{O}{\overset{||}{C}}-O-$ Gua ... Gua
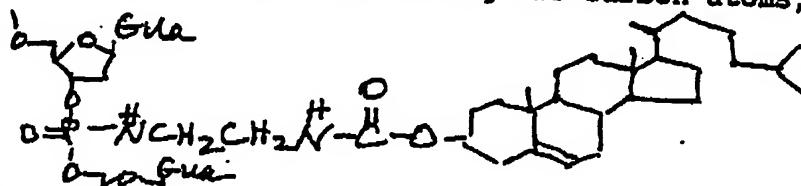
...N N'... internucleoside link with a cholesteryl group tethered
    chol   by a linker containing ten carbon atoms, as in ..G G..
   chol

$O=\overset{\overset{O}{|}}{\underset{\underset{O}{|}}{P}}-N-(CH_2)_{10}N-\overset{O}{\overset{||}{C}}-O-$
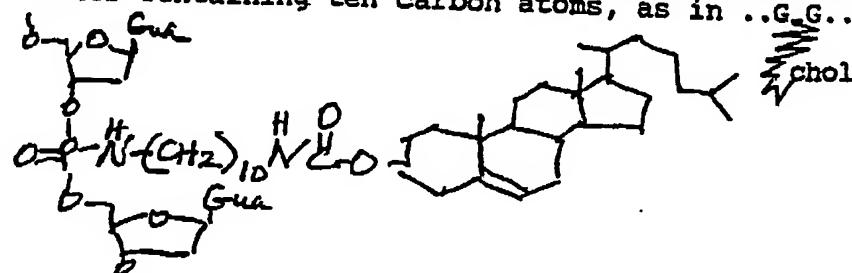
CholesterylsN.. Terminal cholesteryl as in CholesterylsC...

$O=\overset{\overset{O}{|}}{\underset{\underset{O}{|}}{P}}-S^-$ Cyt+

CholesterylpC...

$O=\overset{\overset{O}{|}}{\underset{\underset{O}{|}}{P}}-O^-$ Cyt+

StigmasterylsC...

C_2H_5

$O=\overset{\overset{O}{|}}{\underset{\underset{O}{|}}{P}}-O^-$ Cyt+
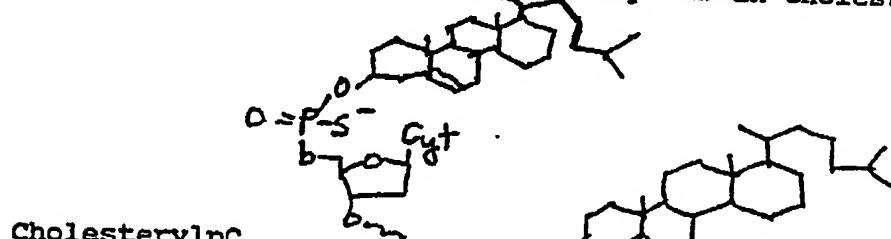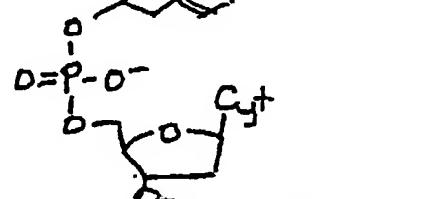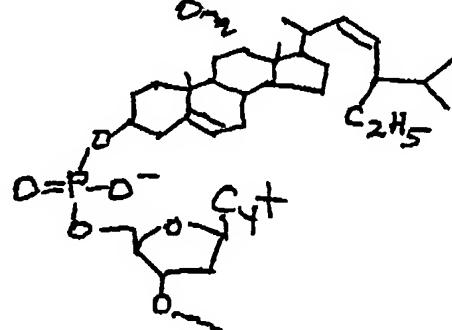
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EXPERIMENTATION

All compounds were synthesized on
controlled pore glass supports using a Biosearch 8600
5   DNA synthesizer or a syringe for manual manipulation.
Internucleoside phosphorothioate links in compounds
1-4, 6, 8-16 were generated by conventional hydrogen
phosphonate chemistry using 5'-
dimethoxytritylnucleosides and terminal oxidation of
10   all hydrogen phosphonate links with sulfur.
Cholesteryl was joined to the chain in 1-4, 8, 10,
12, 14-16 by oxidative compling of 2-
(cholesteryloxycarbonylamino)-ethylamine at an
internucleoside hydrogen phosphonate link via
15   reaction with carbon tetrachloride. The procedure
for tethering cholesteryl in compounds 11 and 13 was
identical except that 2-(cholesteryl-
oxycarbonylamino)-decylamine was used as the amine
component. All these procedures are described in the
20   original patent application and/or reference 1. For
synthesis of 6, cholesteryl was joined utilizing
cholesteryl H-phosphonate. For the synthesis of
compounds with phosphodiester links 5' to
phosphorothioate links (compounds 5 and 7), chains
25   were built using methyl phosphoramidate coupling
followed by oxidation with sulfur at each step. W. J.

Stec, et al, Am. Chem. Soc., 106:6077 (1984). The terminal cholesteryl phosphate was added by oxidative coupling with cholesteryl H-phosphonate and carbon tetrachloride, as for compound 6, followed by

5   standard oxidation with iodine/water. Treatment with thiophenol to remove the methyl protecting groups and ammonium hydroxide to remove base protecting groups and cleave the oligomer from the solid support the afforded the modified oligonucleotide.

10   Compounds 15, 16 and a related substane, compound 17 with the structure XsXsXsXsXsXsXsXsX chol T, were prepared using the syringe technique and DMT-OCH$_2$CH$_2$CH$_2$OP(O)(H)O$^-$ (compound 18 in place of a nucleoside H-phosphonate. For synthesis of 18, 1,3-

15   propanediol (75 mmol) was stirred with dimethoxytrityl chloride (15 mmol) in dry pyridine (25 ml) for about 20 hours at room temperature. Addition of water (100 ml), extraction with chloroform, and chromatography on a silica gel column

20   afforded 3-dimethoxyltrityloxypropanol as a viscous oil; 56% yield; NMR in CDCl$_3$: 1.85 (p, 2H, -CH$_2$-), 2.21 (broad t, 1H, 0-OH), 3.28 (broad t, 2H, -CH2(OH), 3.79 (broad t, 8H, two CH$_3$O-), 6.82 (q, 4H), 7.21-7.42 (m, 9H aromatic). This alcohol (6.9 mmol),

25   in dry acetonitrile (100 ml) was added dropwise over a period of 1 hour to phosphitilating solution

prepared from phosphorus trichloride (30 mmol),
imidazol (98 mmol), and triethylamine (104 mmol) in
acetonitrile (100 ml) at ice-bath temperature. After
one hour of stirring the flask was allowed to warm to
5   room temperature and to stand for two hours. Water
(50 ml) was added and the mixture was concentrated
under reduced pressure and extracted with chloroform.
Chromatography on silica gel afforded 20.0 g (62%) of
18 as the triethylammonium salt; mp 132-135°C; NMR
10   (CDCl$_3$): ppm 1.22 (t, 9H), 1.91 (p, 2H), 2.93 (q,
6H), 3.13 (t, 2H), 3.72 (S, 6H), 3.97 (t,2H), 6.05
and 7.57 (singlets each, 1H), 6.77 (q, 4H), 7.14-7.39
(m, 9H).

Referring to Table 4, compound 1 is
15   complimentary to a region in HIV-I coating for a
splice site for the TAT gene. Compound 3 is
complimentary to a region for REV gene. Compound 4
is complimentary to a region for NEF gene.

Compounds 2, 5, 6, 7, 8 and 9 are all based
20   on sequence 1, with variations in position, mode of
attachment, and the structure of the lipophilic
steriod conjugate.

Compounds 10-14 are conjugates of
homopolymers of nucleotides.

Referring to the activites shown in Table 4, compounds 1, 3, and 4, which are complimentary to various regions of the HIV-I virus, all show unusually high antiviral activity. These compounds

5   may function by an antisense mechanism. Compound 3 is the most active cholesteryl-conjugated oligonucleotide that has been investigated.

Oligonucleotide phosphorothioate derivatives bearing two cholesteryl groups, one

10   tethered at an internal position, are active, as demonstrated by compounds 2 and 14. Activity for an oligonucleotide with cholesteryl groups tethered at the 5′ and 3′ terminal internucleoside positions as set forth above it has been reported.

15   Cholesteryl conjugates with mixed phosphorothioate and phosphodiester backbones are active as demonstrated by compounds 2, 5, and 7. As shown by compounds 5, 6, and 7, oligomers with cholesteryl attached at the 5′ end, either by a

20   phosphodiester or a phosphorothioate link, are active. The activity is not strongly dependent on which of these links is used; that is, ID50 is 7 ug/ml for the phosphorothioate, compound 6, while is it 12 ug/ml for the phosphodiester, compound 7.

Linkage by the carbonylaminoethylamidate, such as in compound 8, affords a more active compound than linking by a phosphodiester, such as compound 7, or a phosphorothioate, such as compound 6.

5   Other highly lipophilic groups, such as stigmasteryl (compound 9) are comparable to cholesteryl in enhancing the antiviral activity of the oligonucleotide derivatives.

Cholesteryl conjugated homooligomers

10   containing thymidine, such as compounds 10, and 11, or deoxycytidine, such as compounds 12, and 13, are active antiviral pharmaceutical compositions. The deoxycytidine oligomers are somewhat active than the thymidine derivatives.

15   Tethers between the steriod and the oligonucleotide having long carbon chains of for example 10 methylene groups, as well as short carbon chains of 2 methylene groups can be used in linking the cholesteryl to the phosphorus atom, as shown by

20   comparing data for the thymidine derivatives (compounds 10 and 11) and the deoxycytidine derivatives (compounds 12 and 13. The compounds with the linker $-NH(CH_2)_{10}NHCO-$ exhibit about the same activity as the corresponding compounds with the

25   linker $-NH(CH_2)_2NHCO-$.

In view of the above, applicant has demonstrated the antiviral activity of the pharmaceutical composition of an oligonucleotide conjugated to a steriod. Applicant has shown that
5 the oligonucleotide can be a homooligomer as well as a specific nucleotide sequence complimentary to various regions of a virus, such as the HIV-I virus. Applicant has also shown that various steriods conjugated to the oligomer have antiviral activity.

10   The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

15   Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims wherein reference numerals are merely for
20 convenience and are not to be in any way limiting, the invention may be practiced otherwise than as specifically described.

Table 2. Inhibition of HIV by Oligonucleotides with Cholesteryl Substituents

compound	conc. µg/ml	% inhibition			ID$_{50}$ µg/ml (syncytia)
		syncytia	P24	RT	
1 (control)	0.16	0			> 100
	0.8	3			
	4	26			
	20	34			
	100	45			
2	2	0	0		10
	5	4	0	0	
	10	51	63	48	
	20	95	88	90	
	50	100	100	92	
3	2	0	0	0	16
	5	2	13	0	
	10	22	70	0	
	20	77	69	0	
	50	100	100	84	
	100	100	100	100	
4	2	0	0	0	32
	5	3	0	0	
	10	7	0	0	
	20	28	32	26	
	50	85	88	75	
	100	100	100	100	
5	2	0	0	0	11
	5	5	0	0	
	20	92	100	82	
	50	100	100	100	
	100	100	100	100	

Table 3. Inhibition of HIV by Phosphorothioate Oligonucleotides with Cholesteryl Substituents

compound	conc. μg/ml	% inhibition				ID$_{50}$ (syncytia)
		syncytia	P17	P24	RT	
6 (phosphodiester control)	2.5	15	13	22	33	6.0
	6.25	56	67	81	70	
	10	90	89	85	85	
	25	100	100	100	100	
7	0.25	0	12	19	23	0.8
	1.0	74	69	70	68	
	1.5	100	100	100	100	
	6.0	100	100	100	100	
8 (phosphodiester control)	1.6	0	0	0	0	14.5
	6.25	15	16	26	26	
	25	95	84	82	67	
	100	97	96	96	78	
9	1.6	25	35	43	47	3.2
	6.25	98	92	96	73	
	25	98	96	96	76	
	100	98	96	96	88	
10 (phosphodiester control)	1.6	0	0	0	0	>100
	6.25	0	4	4	0	
	25	0	20	22	25	
	100	0	24	33	28	
11	1.6	30	42	47	45	3.5
	6.25	97	86	88	61	
	25	97	92	92	70	
	100	98	92	96	89	
12	1.6	28	33	40		3.4
	6.25	93	60	67		
	25	100	100	100		
	100	100	100	100		
13	1.6	20	23	31		3.6
	6.25	89	56	67		
	25	100	100	100		
	100	100	100	100		
14	1.6	18	21	25		13
	6.25	35	30	32		
	25	90	70	66		
	100	100	100	100		
15	25	80	20	25		
	50	99	99	90		
	100	100	100	95		

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Table 4 New Modified Oligonucleotides

Compound	Structure	ID50 (ug/ml)[a]
1[b]	AsCsAsCsCsCsAsAsTsTsCsTsGsAsAsAsAsTsG⌐G ⌐chol	0.4-0.7
2	AsCsAsCsCsCsAsAsTsTsCsTsGsAsAsAsA⌐TpG⌐G ⌐chol⌐chol	1.7
3	GsTsGsTsCsTsCsCsCsCsTsTsCsTsTsCsCsTsGsCsGA⌐T ⌐chol	0.05
4	AsGsTsCsCsAsTsTsGsGsTsCsTsTsAsAsAsGsGsTsAsCsCsCsTsGsAsG⌐G G ⌐chol	0.3
5	CholesterylpAsCsAsCsCsCsAsAsTsTsCsTsGsAsAsAsAsTsGsG	1.5
6	CholesterylsCsTsGsAsAsAsAsTsGsG	7
7	CholesterylpCsTsGsAsAsAsAsTsGsG	12
8	CsTsGsAsAsAsAsTsG⌐G ⌐chol	1.5-2.5
9	StigmasterylsCsTsTsGsAsAsAsAsTsGsG	7-14
10	TsTsTsTsTsTsTsTsT⌐T ⌐chol	10-18
11	TsTsTsTsTsTsTsTsT⌐T ⌐chol	15-30
12	CsCsCsCsCsCsCsCsC⌐C ⌐chol	6
13	CsCsCsCsCsCsCsCsC⌐C ⌐chol	7
14	CsCsCsCsCsCsC⌐CsC⌐C ⌐chol⌐chol	1.7 (toxic > 4 ug/ml)

a. A range in activity is shown for compounds tested more than once.
b. This compound, reported previously, is included for reference.

What is claimed is:

    1.   An oligonucleotide comprising:
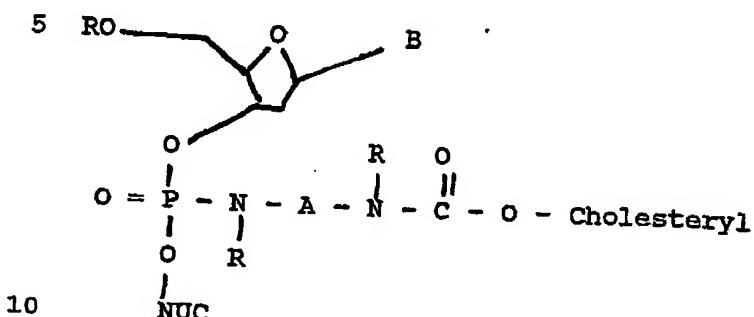
wherein A is selected from the groups consisting of
an aliphatic alkyl, branched aliphatic alkyl and a
heteroatom containing an alkyl (branched) chain of 2
to 18 carbon atoms, R is selected from the group
consisting of H and lower alkyl up to 12 carbon
atoms; NUC is selected from the group consisting of
an oligonucleotide and a phosphothioate
oligonucleotide and B is a base.

    2.   An oligonucleotide as set forth in
claim 1 wherein said oligonucleotide includes 7 to 20
nucleotides.

3. A pharmaceutical composition consisting essentially of: an oligonucleotide and at least one steriod conjugated to said oligonucleotide for increasing the antiriral activity of said
5 oligonucleotide.

4. A composition as set forth in claim 3 wherein said steriod means is cholesterol conjugated to said oligonucleotide.
10

5. A composition as set forth in claim 4 wherein said oligonucleotides include phosphodiester bonds.

15 6. A composition as set forth in claim 5 including phosphothiate substitutions of phosphodiester bonds.

7. A composition as set forth in claim 3
20 wherein said oligonucleotide is complementary to a nucleotide region of a virus.

8. A composition as set forth in claim 7 wherein the virus is HIV-1 virus.
25

9.   A composition as set forth in claim 8 wherein said region of the HIV-1 virus is the TAT gene, the REV gene, or the NEF gene.

5   10.   A composition as set forth in claim 3 wherein said steriod is stigmasteryl.

11.   A composition as set forth in claim 3 including two steriods conjugated to said
10   oligonucleotide.

12.   A composition as set forth in claim 3 wherein said oligonucleotide is a homopolymer.

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT CL. (5): A61K 31/00; C07H 15/00
U.S. CL: 514/45,46,49.50; 536/27,29

II. FIELDS SEARCHED

Minimum Documentation Searched [4]	
Classification System	Classification Symbols
U.S.	514/45,46,49,50; 536/27,29

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [*]	Citation of Document, [16] with Indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A,P	Proceedings of the National Academy of Science, Vol. 86, — September 1989, Robert L. Letsinger et al., "Cholesterylconjugated oligonucleotides: Synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture" pages 6553-6556.	1-12

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the International filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the International filing date but later than the priority date claimed

"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
28 AUGUST 1990	2 4 SEP 1990
International Searching Authority [1]	Signature of Authorized Officer [20]
RO/US	JAMES O. WILSON

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